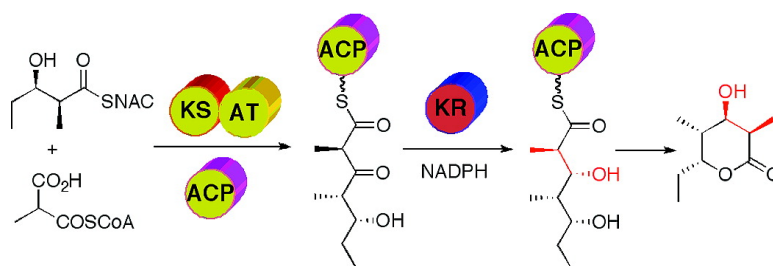


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Stereospecificity of Ketoreductase Domains 1 and 2 of the Tylactone Modular Polyketide Synthase

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Tylactone synthase (TYLS) from *Streptomyces fradiae* is a modular polyketide synthase (PKS) that catalyzes the formation of tylactone (**1**), the parent 16-membered ring macrolactone aglycone precursor of the important veterinary antibiotic tylosin used in the treatment of pulmonary infections in large animals. TYLS consists of seven homodimeric modules, organized into five large proteins of subunit M_D ranging from 170 to 460 kDa, each responsible for a single round of polyketide chain extension and functional group modification (Figure 1).¹

Although the general organization of the tylactone synthase has many features in common with the well-studied modular PKS of erythromycin biosynthesis, 6-deoxyerythronolide B (**2**, 6-dEB) synthase (DEBS),² there are several interesting differences. (1) Tylactone, in common with all other C-14 substituted 16-membered ring macrolides, is derived from an *anti*-diketide intermediate, in this case (2*R*,3*R*)-2-methyl-3-hydroxypentanoyl-ACP generated by TYLS module 1 (Figure 1A). In support of this role is the reported intact incorporation of the corresponding *N*-acetylcysteine analogue **3** into tylactone by cultures of *S. fradiae*³ as well as the observed diastereospecific reduction of racemic 2-methyl-3-keto diketide-SNAC **4** to the *anti*-(2*R*,3*R*)-diketide **3** catalyzed by recombinant TYLS ketoreductase 1 (KR1) (Scheme 1).⁴ By contrast, 12- and 14-membered ring macrolides are derived from an initially generated *syn*-(2*S*,3*R*)-diketide (Figure 1B).^{2,4,5} (2) Tylactone carries a trisubstituted double bond generated by the combined action of the KR2 and DH2 domains of TYLS module 2, unlike 6-dEB which has no double bonds. The predicted mode of action of TYLS module 2 is supported by the intact incorporation of the corresponding unsaturated triketide-SNAC **5** into tylactone.³ Although the stereochemistry of the corresponding β -hydroxyacyl-ACP triketide intermediate is necessarily cryptic, a (3*R*)- β -hydroxy configuration is predicted based on the presence of a conserved active site LDD sequence in the TYLS reductase KR2.^{6,7} We now report the determination of the intrinsic stereospecificity of the KR domains from TYLS modules 1 and 2 and provide the first experimental evidence that the DH domain of a modular PKS catalyzes a *syn* dehydration.

Incubation of recombinant TYLS KR1, expressed and purified as previously described,^{7b} with recombinant DEBS [KS3][AT3] didomain and [ACP3]^{2,8,9} in the presence of *syn*-diketide **6**, methylmalonyl-CoA, and NADPH, followed by basic hydrolysis and acidification, as previously described,^{9,10} gave the *anti*-(2*R*,3*R*)-2-methyl-3-hydroxytriketide lactone **7** as the exclusive product

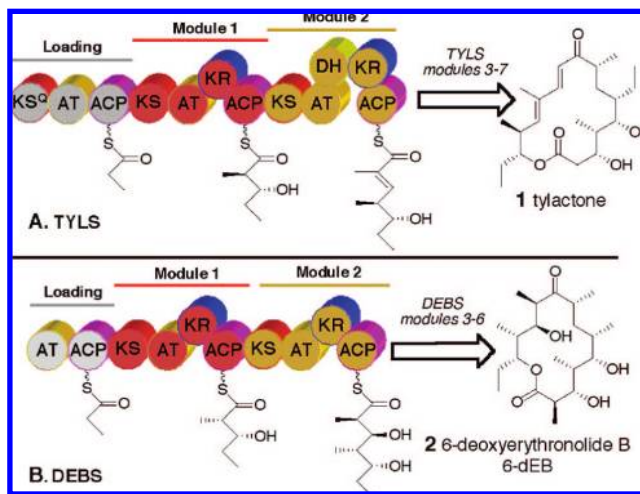
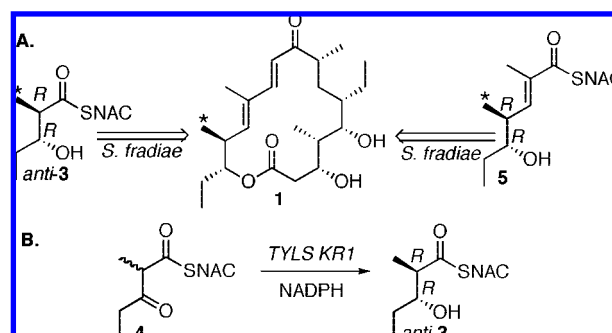


Figure 1. Modular organization of (A) tylactone synthase (TYLS) and (B) 6-deoxyerythronolide B synthase (DEBS); the loading and first two extension modules of each PKS are shown. In addition to the three core catalytic domains—the β -ketoacyl-ACP synthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains—individual extension modules carry specific combinations of tailoring ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER, not shown) domains.

Scheme 1^a



^a (A) Incorporation of [2'-¹³C]-**3** and [4'-¹³C]-**5** into tylactone (**1**). (B) Diastereoselective reduction of racemic ketoester **4** to *anti*-(2*R*,3*R*) **3** by recombinant TYLS KR1.

(Scheme 2). The configuration of **7** was unambiguously assigned by capillary GC–MS analysis of the corresponding **7**-TMS derivative and direct comparison with synthetic standards of each of the four diastereomeric triketide lactones.¹⁰ The same triketide lactone diastereomer **7** was also obtained by an incubation using DEBS [KS6][AT6] plus [ACP6] in combination with TYLS KR1.

TYLS KR2 differs from other type I PKS KR domains that have previously been cloned and expressed in active form in that it is

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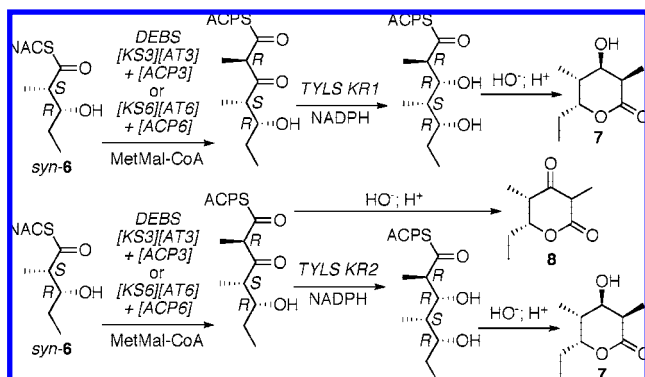
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Scheme 2. Stereochemistry of Triketide Lactone Formation Catalyzed by TYLS KR1 and KR2 in Combination with Dissected DEBS [KS][AT] and [ACP] Domains



bounded immediately upstream by a DH rather than an AT domain. The conserved *N*-terminal YRVEW and *C*-terminal RLAGL boundaries of the KR2 domain^{2,4,6a,7b,9} were identified by multiple sequence alignments, and the corresponding PCR primers were used to amplify the intervening KR2 domain using DNA originally derived from plasmid pKOS168–190 harboring the entire TYLS gene cluster as a template. The resultant amplicon was inserted into pET28a to give pRC18 encoding TYLS KR2 with an appended *N*-terminal His₆-tag. Unexpectedly, recombinant KR2 was found to be catalytically inactive. Multiple sequence comparisons, however, reveal that TYLS KR2 has a Ser-Gly diad in place of the universally conserved Ser-Ser sequence in which the second Ser is an essential part of the active site Ser-Tyr-Lys triad typical of short chain dehydrogenase-reductases.^{6a,11,12} We therefore replaced Gly365 with a Ser by site-directed mutagenesis.¹³ Incubation of Ni-NTA-purified TYLS KR2(G365S) with either DEBS [KS3][AT3] and [ACP3] or DEBS [KS6][AT6] and [ACP6] in the presence of diketide **6**, [2-¹⁴C]methylmalonyl-CoA, and NADPH gave a mixture of the triketide ketolactone **8** and the lactone **7**, as established by TLC-phosphorimaging. GC–MS analysis of the corresponding 7-TMS derivative (ret. time 7.01 min) established the formation of lactone **7** as the exclusive reduction product (Scheme 2).

We have previously determined that the ACP-bound 2-methyl-3-ketoacyl triketide intermediates generated by both DEBS [KS3][AT3] and [KS6][AT6] have exclusively the *D*-methyl (2*R*) configuration at C-2, as established by trapping with NaBH₄.¹⁰ The finding that both TYLS KR1 and KR2 reduce this intermediate to give a single *anti*-(2*R*,3*R*,4*S*,5*R*)-2,4-dimethyl-3,5-dihydroxyheptanoyl-ACP product corresponding to triketide lactone diastereomer **7** is consistent with the established generation of the *anti*-diketide by the KR1 domain of TLYS module 1. This report is also the first demonstration that the KR1 domain can reduce polyketides longer than the natural diketide 3-ketoacyl thioester substrate. Since recombinant TYLS KR2 is expected to retain the intrinsic diastereoselectivity of the integrated KR2 domain, these results establish for the first time that the *anti*-triketide product is generated by TYLS KR2 as part of the catalytic sequence mediated by TLYS module 2. The subsequent dehydration catalyzed by TYLS DH2 to give

(2*E*,4*S*,5*R*)-2,4-dimethyl-5-hydroxyhept-2-enoyl-ACP2 (Figure 1A) must therefore involve a *syn* dehydration, consistent with the known *syn* stereospecificity of the yeast fatty acid synthase DH domain.¹⁴ Interestingly, the generation of a *D*-β-hydroxyl group by TYLS KR2 (*re* specificity) contrasts with that of DEBS KR2 which generates the *syn*-(2*R*,3*S*,4*S*,5*R*)-triketide en route to 6-deB (Figure 1B)¹⁰ but is identical to the *D*-(3*R*)-β-hydroxyl specificity that we have previously established for the picromycin synthase KR2 and the rapamycin KR4 domains, each of which is paired with a DH domain.¹⁵

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Supporting Information Available: Experimental procedures, GC–MS data, and sequence alignments. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- (13) The resulting TYLS KR2(G365S) reduced the standard model substrate *trans*-1-decalone, with k_{cat} 0.27 ± 0.02 s⁻¹ and k_{cat}/K_m 800 ± 270 M⁻¹ s⁻¹ (cf. k_{cat} 0.12–0.28 s⁻¹, k_{cat}/K_m 10–190 M⁻¹ s⁻¹ for recombinant DEBS KR domains, ref 4).
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